Please amend claim 29 as follows:

29. (amended) A plasmid comprising a nucleic acid sequence that encodes a modified *Mth* RIR1 intein, wherein said plasmid is selected from the group consisting of pMRB8P, pMRB8A, pMRB8G1, pMRB9GS, pMRB9GA, pMRB10G and pBRL-A.

A "clean copy" of revised claims 2, 8, 16, 17, 28, and 29 is attached.

REMARKS

The present invention relates to a method for the ligation of expressed proteins utilizing one or more inteins which display cleavage at their N- and/or C-terminal.

35 U.S.C. §112 CLAIM REJECTIONS

Claims 1-4, 6, 8-12, 15-19, 21, 24, 25, 27 and 28 were rejected under 35 U.S.C. §112, first paragraph, the Examiner taking the position that because the specification, while being enabling for methods for fusing target proteins initially generated by cleavage of intein-comprising precuror proteins wherein a second target protein, or region, in the method has an amino-terminal cysteine, and for resulting, ligated, fusion protein, whether linear, cyclic, or polymeric, does not reasonably provide enablement for methods for fusing target proteins, or for

the resulting fusion polypeptides, wherein a second target protein used in a fusion method has no amino-terminal cysteine. The Examiner asserts that the specification does not enable any person skilled in the art to which it pertains, or with which it most nearly connected, to make and use the invention commensurate in scope with these claims. Applicants respectfully disagree.

Applicants would like to bring to the attention of the Examiner two references published subsequent to Applicants' priority date which demonstrate successful ligation of a peptide or protein that did NOT have an N-terminal cysteine to a second thioester-tagged protein. These references clearly demonstrate the fact that a peptide without an N-terminal cysteine can be used to ligate to a second peptide with a C-terminal thioester substantially in accordance with the teaching of the present Application.

Specifically, the first reference, Canne, et al., "Extending the Applicability of Native Chemical Ligation", *J. Am. Chem. Soc.* 118:5891-5896 (1996), (copy attached), uses N^{α}(ethanethiol) or N^{α}(oxyethanethiol) peptides to ligate to a second peptide containing a C-terminal thioester. These peptides do not have an N-terminal cysteine and the authors demonstrated that a derivative of glycine or alanine at the N-terminus of a peptide could result in a ligation product with glycine or alanine, respectively, at the site of ligation (instead of a cysteine).

The second reference, Hondal, et al., "Selenocysteine in native chemical ligation and expressed protein ligation", *J. Am. Chem. Soc.* 123:5140-5141 (2001), (copy attached), describes how an N-terminal selenocysteine (a naturally coded amino acid) can be used in place of an N-terminal cysteine for ligation to a second protein or peptide with a C-terminal thioester.

Yet, another reference, Nilsson, et al., "Staudinger Ligation: A peptide from a thioester and azide" *Org. Lett.* 2:1939-1941 (2000), (copy attached), starts with a thioester-tagged protein as in the methodology described in the instant invention and modification of the thioester by reacting it with a phosphinothiol. The resulting phosphinothioester-tagged protein is then ligated to a peptide with an N-terminal azide. Concerning the requirement for an N-terminal cysteine using this method, the authors themselves state "Here, we describe a method for peptide ligation that eliminates the need for a cysteine residue and leaves no residual atoms in the peptide product."

Accordingly, contrary to the Examiner's position, Applicants respectfully submit that the present specification enables and is commensurate in scope with the present claims. The rejection should therefore be withdrawn.

Claims 2-14, 16-20, 28 and 29 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to

particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Applicants have amended the claims 2, 8, 16, 17, 28 and 29 as suggested by the Examiner. Accordingly, this rejection should be withdrawn.

PRIOR ART REJECTIONS

A. 35 U.S.C. §102 - CLAIM REJECTIONS

Claims 1 and 15 were rejected under 35 U.S.C. §102(a) as being anticipated by Muir, et al., *Proc. Natl. Acad. USA* 95:6705-6710 (1998).

Claims 1 and 15 were also rejected under 35 U.S.C. §102(a) as being anticipated by Severinov, et al., *The Journal of Biological Chemistry* 273:16205-16209 (1998).

Claims 24 and 28 were rejected under 25 U.S.C. §102(a) as being anticipated by Chong, et al., *The Journal of Biological Chemistry* 273:10567-10577 (1998).

B. 35 U.S.C. §103 - CLAIM REJECTIONS

Claims 2, 7, 8-10 and 14 were rejected under 35 U.S.C. §103(a) as being unpatentable over either of Muir, et al., or Severinov, et al., disclosed above, in view of Comb, et al., U.S.

Patent No. 5,496,714 and Mills, et al., *Proc. Natl. Acad. Sci. USA* 95:3543-3548 (1998).

With respect to the §102(a) rejections, none of these references pre-date by more than one year Applicants claimed priority date of Provisional Application Serial No. 60/102,413.

With respect to the rejections of claims 1 and 15, Applicants are submitting herewith a Declaration under 37 C.F.R. 1.131 by Dr. Thomas C. Evans, one of the inventors, which establishes that the subject matter of claims 1 and 15 was invented by the inventors prior to effective date of the Muir, et and Severinov, et al. references relied on by the Examiner. See ¶5 of the Evans Declaration.

Specifically, ¶¶6-10 describe a ligation reaction whereby a truncated thioester-tagged RnaseA protein using the modified intein described on pages 90-91 of Dr. Evan's Notebook 4 (attached as Exhibit A to his Declaration) is ligated to a 15 amino acid synthetic peptide that is identical to amino acid residues 100-124 of RnaseA which resulted in a full-length RnaseA protein product with an N-terminal cysteine (Evans Declaration at ¶8).

The gel on page 104 of Dr. Evans' Notebook (lane 7) depicts the ligation product (Evans Declaration, ¶10).

This clearly demonstrates that Applicants had invented the subject matter of claims 1 and 15 prior to the effective date of

the Muir, et al. and Severinov, et al. references (Evans Declaration, ¶10).

With respect to the §102(a) rejection of claims 24 and 28, over Chong, et al., Applicants are submitting herewith a Petition to Correct Inventorship by adding Dr. Shaorong Chong as co-inventor of claims 24 and 28. The addition of Dr. Chong resulted from Applicants review of the ¶102(a) rejection over Chong, et al. As set forth in the statement attached to the Petition, this error occurred without deceptive intent on the part of Dr. Chong. As the inventors and all of the authors of Chong, et al. were employed by the Assignee at all relevant times, the invention claimed was not "before the invention by the Applicant for a patent." The non-inventor co-authors of the Chong, et al. reference, namely Kay S. Williams and Chad Wotkowicz, worked under the direction of Dr. Chong. Their contribution did not rise to the level of inventorship. Applicants would be glad to submit a Declaration to this effect if requested by the Examiner.

Accordingly, the §102(a) rejections should be withdrawn.

Similarly, the rejection under §103 should be withdrawn as the primary references relied on by the Examiner are the above-referenced Muir, et al. and Severinov, et al. references. It is submitted that the Declaration by Dr. Evans overcomes this rejection.

CONCLUSION

For the reasons set forth above, Applicants respectfully request that the rejections set forth in the Official Action of July 13, 2001 be withdrawn, and submit that this case is in condition for immediate allowance. Early and favorable consideration leading to prompt issuance of this Application is earnestly solicited.

Should the Examiner wish to discuss any of the remarks made herein, the undersigned attorney would appreciate the opportunity to do so. Thus, the Examiner is hereby authorized to call the undersigned attorney collect at the number shown below.

Respectfully submitted,

NEW ENGLAND BIOLABS, INC.

Dated: 1/10/02

Gregory D. Williams (Reg. No. 30901)
Attorney for Applicants
32 Tozer Road
Beverly, Massachusetts 01915
(978) 927-5054 X:292

Customer No.: 28986

CLEAN-COPY OF REVISED CLAIMS

2. The method of claim 1, wherein said first target protein of step (a) is generated from a first plasmid comprising at least one nucleic acid sequence that encodes a first intein having N-terminal cleavage activity and said second target protein of step (b) is generated from a second plasmid comprising at least one nucleic acid sequence that encodes a second intein having C-terminal cleavage activity.

8. A method for fusion of expressed proteins, said method comprising the steps of:

(a) constructing a first plasmid comprising at least one nucleic acid sequence that encodes a first modified intein, wherein said first modified intein is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of said first target protein;

(b) constructing a second plasmid comprising at least one nucleic acid sequence that encodes a second intein having C-terminal cleavage activity, wherein said second intein is capable of cleavage to produce a said second target protein having a specified N-terminal;

(c) expressing at least one C-terminal thioester-tagged first target protein from said first plasmid of step (a);

(d) expressing at least one second target protein having a specified N-terminal from said second plasmid of step (b); and

(e) ligating said first target protein of step (c) with said second target protein of step (d).

16. A method for cyclic fusion of an expressed protein, said method comprising the steps of:

(a) constructing a plasmid comprising at least one nucleic acid sequence that encodes a target protein, at least one nucleic acid sequence that encodes a first intein having N-terminal cleavage activity, and at least one nucleic acid sequence that encodes a second intein having C-terminal cleavage activity, wherein said first intein is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of said target protein and wherein said second intein is capable of cleavage to produce a specified amino acid at the N-terminal of said target protein;

(b) expressing a C-terminal thioester-tagged target protein having a specified amino acid at its N-terminal from the plasmid of step (a); and

(c) ligating the N-terminus of said target protein to the C-terminus of said target protein to produce a cyclic protein.

17. A method for polymerization of an expressed protein, said method comprising the steps of:

(a) constructing a plasmid comprising at least one nucleic acid sequence that encodes a target protein, at least one nucleic acid sequence that encodes a first intein having N-terminal cleavage activity, and at least one nucleic acid sequence that encodes a second intein having C-terminal

cleavage activity, wherein said first intein is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of said target protein and wherein said second intein is capable of cleavage to produce a specified amino acid at the N-terminal of said target protein;

(b) expressing a C-terminal thioester-tagged protein having a specified amino acid at its N-terminal from the plasmid of step (a); and

B3 Cont

(c) intermolecular ligation of said target proteins to yield a protein polymer.

28. A plasmid comprising at least one nucleic acid sequence that encodes a modified intein of any one of claims 22-27.

B4

29. A plasmid comprising a nucleic acid sequence that encodes a modified *Mth* RIR1 intein, wherein said plasmid is selected from the group consisting of pMRB8P, pMRB8A, pMRB8G1, pMRB9GS, pMRB9GA, pMRB10G and pBRL-A.

Docket No.: NEB-154

IN THE UNITED SATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Evans	EXAMINER:	W. Moore
APPLICATION NO.:	09/249,543	GROUP:	1652
FILED:	February 19, 1999		
FOR:	Intein-Mediated Protein Ligation Of Expressed Proteins		

The Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

DECLARATION UNDER 37 C.F.R. §1.131

As a below named inventor, I hereby declare that:

1. I am one of the named inventors on the above-identified Application.

2. I understand that claims 1 and 15 were rejected under 35 U.S.C. §102(a) as being anticipated by Muir, et al., *Proceedings of the National Academy of Sciences. USA* 95:6705-6710 (1998).

3. I understand that claims 1 and 15 were rejected under 35 U.S.C. §102(a) as being anticipated by Severinov, et al., *The Journal of Biological Chemisty* 273:16205-16209 (1998).

4. I understand that claims 2, 7, 8-10 and 14 were rejected under 35 U.S.C. §103(a) as being unpatentable over either Muir, et al. or Severinov, et al., in view of Comb, et al., U.S. Patent No. 5,496,714, and Mills, et al., *Proceedings of the National Academy of SciencesUSA* 95:3543-3548 (1998).

5. Attached hereto as Exhibit A are pages 90-92 and page 104 from my notebook (Notebook No. 4). The dates reported therein have been redacted. The work reported on these pages was completed prior to June of 1998, the effective publication date of Muir, et al. and Severinov, et al.

6. Pages 90-91 of my notebook describe plasmid DNA isolated from T1 transformed into *E. coli* strain ER2566. This plasmid DNA expresses the truncated RNase A-*Mxe* GyrA intein-chitin binding domain fusion protein. The transformed strain was termed T2.

7. Pages 90-91 of my notebook also describe the induction of protein expression from T2. Following induction, the *E. coli* cells were pelleted, resuspended and lysed by sonication. The sonicated solution was subjected to centrifugation and this clarified cell lysate was referred to as the supernatant or supe. The supe was applied to a chitin resin and a sample of the material that passed through the column was saved. The saved sample was termed the flowthrough or FT. The truncated RNase A-*Mxe* GyrA intein-chitin binding domain fusion protein binds to the chitin

column. The column was then equilibrated in a buffer containing MESNA (2-mercaptoethanesulfonic acid) and the column was incubated at 4°C overnight. The next morning fractions were collected from the chitin column. Fractions from the chitin column used for T2 were concentrated using a spin concentrator.

8. Page 92 of my notebook describes a ligation reaction (PL3) using the truncated, thioester-tagged RNase A protein purified using the modified intein tag as described on pages 90-91. The 15mer is a 15 amino acid synthetic peptide that is identical to amino acid residues 110-124 of RNase A. Ligation of this peptide to the truncated, thioester-tagged RNase A resulted in a full-length RNase A protein product. (See Gel 2, lane 7 from page 104 of my notebook and the description of that page hereinbelow.) Note that this peptide has an N-terminal cysteine.

9. Page 104 of my notebook depicts Gel 2, which, is an SDS-PAGE gel of the purification of the truncated, thioester-tagged RNase A and its subsequent use in ligation reactions. Lane 3 is a Broad Range molecular weight marker. The molecular weights of the visible bands, beginning from the top, are 212, 158, 116, 97, 66, 56, 43, 36, 27, 20, 14 and 7 kilodaltons, respectively. Lane 4 is a sample of the clarified cell extract (supe) from the purification using T2. Lane 5 is the clarified cell extract following application to a chitin column. Note that the band of *ca* 41 kDa present in the supe is absent in the flowthrough. Lane 6 is the concentrated fractions from the chitin column. The major band is the correct

size for the truncated, thioester-tagged RNase A protein. Lane 7 is PL3. This lane depicts the PL3 ligation reaction, namely, the truncated, thioester-tagged protein ligated to the 15 amino acid synthetic peptide containing an N-terminal cysteine. This is apparent because of the appearance of a new band migrating with a higher apparent molecular weight. Lanes 8 and 9 are full-length RNase A, either purified by HPLC from another ligation reaction or obtained from the Sigma Chemical Co., respectively. Note that the ligation product from Lane 7 migrates with the same apparent molecular weight as full-length RNase A, as expected. Lane 10 is not germane to this Declaration.

10. These pages from my notebook clearly demonstrate that we had completed the invention embodied in claims 1 and 15 prior to the effective publication dates of Muir, et al. and Severinov, et al.

I further declare under penalty of perjury pursuant to laws of the United States of America the foregoing is true and correct and the Declaration was executed by me on:

Thomas C. Evans

Date 1/10/2002

EXHIBIT A

THOMAS C. EVANS
LABORATORY NOTEBOOK NO. 4
PAGES 90-92 & 104

Project No. _____
Book No. _____ TITLE _____

From Page N .__

T1	T2
50 mL ER2566	50 mL ER2566
2 mL Hpa#6 ①	2 mL T1

1 L of LB ampt broth was inoculated w/ T1 and
3 x 1L " " w/ T2
These were ~~started~~ growing at 37°C at 9:45 AM

The samples were grown to the below OD_{600}'s and then 6 mL of 100 mM IPTG was added.

T1 $OD_{600} = 0.45$
T2 (1) $OD_{600} = 0.63$
T2 (2) $OD_{600} = 0.47$
T2 (3) $OD_{600} = 0.507$

Induction was at 15°C O/N.

(all purification steps were on ice or at 4°C)

The 4 x 1L samples from above were centrifuged at 4000 rpm's in a JS-4.2 rotor for 30 min. The supernatant was poured off and the pellet saved.

The pellet ~~of~~ T1 was resuspended in 2 x 25 mL of Column Buffer

The T2 pellets were resuspended w/ 25 mL of Column buffer (each pellet w/ the same 25 mL) and each rinsed w/ another 25 mL (total volume = 50 mL + the 3 pellets (resuspended))

Column buffer = 50 mM Tris, 500 mM NaCl, pH 7.4

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
		Record d by	

From Page No. —

The resuspended pellets were sonicated (1 x 5 min for T1, 2 x 5 min for T2)

The sonicated samples were applied to chitin columns (10 mL for T1 and 20 mL for T2) at a flow rate of 0.5 mL/min.

Unbound protein was washed off the T1 column w/ 250 mL of Column buffer.

Washing of the T2 column was w/
1) 100 mL Column buffer
2) 100 mL 50 mM Tris, 500 mM NaCl, 0.2% Triton X-100, pH 7.4
3) 150 mL Column buffer

Cleavage was initiated w/ 50 mM Tris, 100 mM NaCl, 100 mM MES, pH 8 and let react O/N at 4°C.

Cleaved protein was eluted from both columns w/ 50 mM Tris, 100 mM NaCl, pH 8.0, 50 mM MESNA
3 mL fractions were collected.
Fractions from T1 were used immediately for ligation (PL1 & PL2)
The 1st 11 fractions were applied to a 5 mL column of chitin to remove any bleeding of the precursor from the original column. This flowthru was concentrated using a centriprep 3 to 1.5 mL (90 min spin → 30 min spin → 15 min spin) original volume was ~27 mL.
This concentrated sample (T2 conc.) was used for ligation (PL3 & PL4)

To Page No. —

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

From Pag No.__

PL1

916 mL T1 Frac 3

0.0036 g 31mer

started @ 10:00 AM

stored on ice or at 4°C

PL2

~~600~~ 685 mL T1 Frac 3

15 mL 1M NH_2OH

Started @ 10:25

stored on ice or at 4°C

PL3

1.22V mL T2 conc.

0.0020 g 15mer

started at 11:38 Am

stored on ice or at 4°C

PL4

194 mL T2 conc

6 mL 1 M NH_2OH

started @ 12:25

stored on ice or at 4°C

To Page No.

Witnessed & Understood by me,	Dat	Inv nt d by	Date
		Rec rded by	

Project No. ______ Book No. ______ TITLE ______

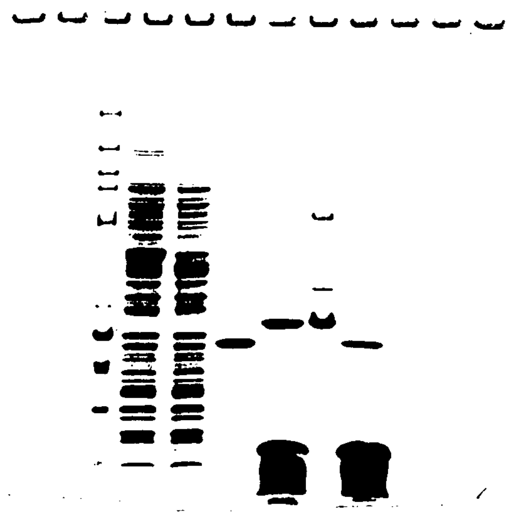
From Page No.

SDS-PAGE gels of RNase A + HpaI for the paper
10-20% Tricine gels

gel 1

lane	
1	3X sample Buffer 3 uL
2	3X sample Buffer 3 uL
3	NEB Broadrange 15 uL
4	T1 supe 10 uL
5	T1 FT 10 uL
6	T1 Frac 3 20 uL
7	PL1 20 uL
8	HpaI wt 20 uL
9	PL1 20 uL
10	3X sample buffer & 3 uL
11	↓
12	3X sample buffer 3 uL

not yours 09

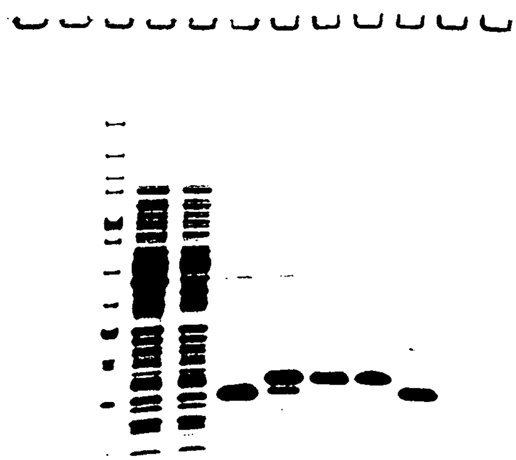


Samples: 14 uL sample + 7 uL 3X sample buffer (w/ DTT)

Tom Evans

gel 2

lane	
1	3X sample Buffer 3 uL
2	3X sample Buffer 3 uL
3	NEB Broadrange 15 uL
4	T2 supe 10 uL
5	T2 FT 10 uL
6	T2 conc 10 uL
7	PL3 10 uL
8	HpaI purified RNase A 10 uL
9	Sigma RNase A 12 uL
10	PL2 10 uL
11	3X sample buffer 3 uL
12	3X sample buffer 3 uL



T2 supe + FT = 3 uL sample + 11 uL H_2O
+ 7 uL 3X sample buffer (w/ DTT)
Sigma RNase A: 0.5 uL Sigma RNase A + 13.5 uL H_2O
+ 7 uL 3X sample buffer
others: 14 uL sample + 7 uL 3X sample buffer (w/ DTT)

Tom Evans

Witnessed & Understood by me.	Date	Invented by	Date
		Recorded by	